Arginine dependence of tumor cells: targeting a chink in cancer's armor

Arginine Dependence of Tumor Cells: Targeting a Chink in Cancer's Armor

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Abstract

Arginine, one among the twenty most common natural amino acids, plays a pivotal role in cellular physiology as it is being involved in numerous cellular metabolic and signaling pathways. Dependence on arginine is diverse for both tumor and normal cells. Due to decreased expression of argininosuccinate synthetase (ASS) and/or ornithine transcarbamoylase (OTC), several types of tumor are auxotrophic for arginine. Deprivation of arginine exploits a significant vulnerability of these tumor cells and leads to their rapid demise. Hence, enzyme-mediated arginine depletion is a potential strategy for the selective destruction of tumor cells. Arginase, arginine deiminase (ADI) and arginine decarboxylase (ADC) are potential enzymes that may be used for arginine deprivation therapy. These arginine catabolizing enzymes not only reduce tumor growth but also make them susceptible to concomitantly administered anti-cancer therapeutics. Most of these enzymes are currently under clinical investigations and if successful will potentially be advanced as anti-cancer modalities.

Keywords: cancer, arginine deprivation, arginase, arginine deiminase, arginine decarboxylase

Introduction

Amino acids play a major role in regulating important cellular events in both normal and malignant cells. Besides their role in the synthesis of hormones and peptides, amino acids also function as cell signaling molecules, playing a modulatory role in gene expression.[1] Amino acids regulate RNA synthesis by diverse mechanisms ranging from regulating transcription factors assembly,[2] to total mRNA turnover.[3,4] Amino acids are major determinants of a normal cellular physiology, therefore potential signaling pathways such as amino acid response (AAR) pathway sense their altered metabolism [Figure 1]. Hence, amino acid levels in the body are critical for important cellular functions.[5-9]

There is a significant difference between the metabolism of normal and malignant cells.[10] For instance, bio-energetic requirements for homeostasis in normal cells are fulfilled by catabolic metabolism. On the other hand, the majority of the tumor cells alter their metabolic program ("*metabolic remodeling*") and consume additional nutrients in order to maintain a balance between elevated macromolecular biosynthesis[11] and adequate levels of ATP for survival.[12,13] However, the endogenous supply of nutrients becomes inadequate during intense growth. Thus tumor cells depend on exogenous nutrients in their microenvironment to fulfill the elevated energy requirements *i.e*. they become auxotrophic for nutrient and energy sources.[14-16] Deprivation of amino acids results in growth inhibition or death of tumor cells by the modulation of various signaling cascades.[6-9,17,18]

Exogenously incorporated enzymes that deprive amino acids could be a novel strategy for the treatment of auxotrophic tumors. The first FDA approved heterologous enzyme for the treatment of cancer was *E. coli* L-asparaginase.[19] L-asparaginase exploits the differences on their dependence of normal and leukemic cells towards L-asparagine.[20] L-asparaginase has been proven to be a promising agent for the treatment of L-asparagine auxotrophic T-cell acute lymphoblastic lymphoma (T-ALL). Use of L–asparaginase in T-

ALL opened up new windows of 'amino acid-depriving therapy'. Currently, there is a resurgence of interest in enzyme-mediated amino acid deprivation as a new therapeutic approach for cancer treatment.[6,7,21,22] For example, arginine depletion can inhibit tumor cell proliferation and induce cell death pathways. Here we endeavor to provide a basic understanding of the roles of arginine in normal and tumor cell with emphasis on current knowledge and developments in the application of enzyme-mediated arginine depriving therapy as a potential anticancer approach.

Enzyme-mediated arginine deprivation: a potential anti-cancer approach

Arginine is involved in the regulation of various molecular pathways and thus availability of arginine can modulate key metabolic, immunological, neurological and signaling pathways of the cells [Figure 2 and 3].[23,24] Auxotrophy towards arginine by certain tumor cells (particularly that of hepatocellular carcinoma and melanoma) has been well characterized.[25,26] Normal cells, when deprived of arginine, undergo cell cycle arrest at G_o/G_1 phase and become quiescent. If reinstated with arginine, the majority of the normal cells recover to their normal proliferation status. However, arginine deprivation in tumor cells does not arrest cell cycle at G_1 phase and continue to be in a cell cycle, leading tumor cells to undergo unbalanced growth and eventually lead to the activation of apoptotic pathways.[27,28]

Owing to the involvement of arginine in a plethora of cellular pathways, arginine dependence of tumor cells has rapidly emerged as a potential target for cancer.[29] However, dietary restriction results in the reduction of only 30% of plasma free arginine.[30] Thus, arginine degrading enzyme-mediated arginine deprivation has been proposed as a potential anti-cancer therapy by various research groups.[27-35] Enzymes that can be used for arginine deprivation therapy (ADT) include arginine deiminase (ADI), arginase and arginine decarboxylase (ADC) as discussed below [Figure 3].

1. Arginine deiminase

Arginine deiminase (ADI) (E.C.3.5.3.6) is a prokaryotic enzyme originally isolated from *Mycoplasma*, which catalyzes an irreversible deimination of the guanidine group of L-arginine to citrulline and ammonium ion.[36] Normal cells are able to convert citrulline into arginine through argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), expression of which are tightly regulated. However, the expression of ASS/ASL is down-regulated in certain tumor cells by unknown mechanisms and these cells are unable to convert citrulline to arginine.[30-33,37] This makes the tumor cells auxotrophic for arginine for their growth and cellular functioning. ADI-mediated arginine deprivation leads to apoptotic cell death, selectively of arginine auxotrophic ASS (-) tumor cells sparing the ASS (+) ADI resistant normal cells[38] [Table 1]. Incidence of ASS deficiency varies depending on the tumor type and expression level of ASS has been proposed as a biomarker for identification of ADI sensitive tumors.[24,25,39-42]

In 1990, Miyazaki and co-workers[43] were the first to report the growth inhibition of *Mycoplasma* infected human tumor cells. The cause of growth inhibition of human tumor cell lines was identified as a ADI produced by *Mycoplasma*. *In vitro* growth-inhibitory dose of *Mycoplasmal* ADI appeared to be 1000 times lower than that of bovine liver arginase. Subsequently in 1992, growth inhibitory activity of ADI was demonstrated in *ASS*-downregulated human melanoma cells.[44] These pioneering studies established ADI as a potential anti-cancer enzyme [Figure 4].

1.1 PEGylated ADI

Being microbial in origin, ADI has serious disadvantages of eliciting strong antigenicity and rapid plasma clearance (half-life of 4 h). To circumvent these limitations, several studies have aimed to extend the plasma half-life of ADI and to minimize its antigenicity. In 1993, Takaku *et al* addressed these problems for the first time by

polyethylene glycol (PEG) modification.[45] Remarkably, PEGylation of *Mycoplasma arginini* ADI enhanced its cytotoxic potential *in vivo* and once a week intravenous injection of PEG-ADI at a dose of 5 U/mouse (10 mg protein/Kg) depleted plasma arginine to an undetectable level at least for a week, whereas native enzyme required 10 daily injections to achieve similar effects. Nevertheless, PEGylation of *Mycoplasma hominis* ADI also resulted in significant enhancement of arginine lowering potential of native *Mycoplasma hominis* ADI.[46,47] Recently, PEGylation and pharmacological properties of an engineered ADI originated from *Pseudomonas plecoglossicida* have been studied. PEGylated *Pseudomonas plecoglossicida* ADI remarkably improves the stystemic half-life (by 11-folds) and found to exhibit superior efficacy than native ADI in depleting plasma arginine.[48]

PEG-ADI has also shown promising outcomes for the treatment of human malignancies. In March 1999, ADI-PEG20, PEGylated recombinant *Mycoplasmal* ADI was approved as an orphan drug by US-FDA for the treatment of HCC and malignant melanomas. Subsequently in July 2005, European Agency for the Evaluation of Medicinal Products (EMEA) granted orphan drug status to ADI-PEG20 for the treatment of HCCs.[49]

ADI-PEG20 is currently undergoing clinical investigation as a randomized double-blind Phase III trial in patients with advanced HCC (NCT 01287585), Phase II studies in patients with ASS-negative metastatic melanoma (NCT 01279967) and Phase II studies in patients with relapsed small-cell lung cancer (SCLC) (NCT 01266018)[50] [Table 2]. Outcomes of the previous clinical studies were also encouraging, achieving response rates of 25% and 47% in melanoma and HCC, respectively [Table 2]. Moreover, grade III and IV toxicities have not been observed in clinical investigations involving ADI-PEG20 in metastatic melanoma and HCC patients.[51,52] Therefore, clinicians are looking forward to the establishment of ADI-PEG20 as a potent anti-cancer modality.

1.2 Tumor sensitivity towards ADI

The auxotrophicity of tumors towards arginine and their sensitivity towards it can be attributed to the lack or reduced expression of ASS in tumors.[25,37-39,53] Notably, numerous tumor cells which are deficient in ASS expression, are sensitive towards ADI treatment [Table 1]. Transfection of an expression plasmid containing human ASS cDNA in HCC and melanoma cells confers severe resistance to ADI treatment compared to ASS-negative cells.[47] Till date, most promising targets for ASS expression dependent ADT identified are human melanoma and HCCs. Other promising targets include malignant pleural mesothelioma (MPM), renal cell carcinoma, prostate cancer, T-ALL and osteosarcoma.[50] However, molecular mechanisms underlying tumor sensitivity towards ADI treatment, by down-regulation of ASS expression in tumor cells, are still elusive. Promoter hypermethylation-dependent silencing of ASS gene is an endorsed mechanism of ASS gene repression.[37,54-56] Methylation frequency of the ASS promoter upto 50-80% level at the CpG loci is documented across a broad range of lymphomas. In contrast, normal lymphoid samples were found unmethylated.[26] Treatment of ADI-PEG20 to *ASS*-methylated lymphoma cell lines revealed dramatic decrease in the proliferation rate and viability count, by inducing caspase-dependent apoptosis, without affecting normal lymphoblastoid cell lines. Demethylation-induced resistance to ADI-PEG20 treatment has also been confirmed in Cutaneous T-cell Lymphoma (CTCL) cell lines, as their incubation with 5-Aza-dC (demethylating agent) for 8 days which resulted in partial demethylation, followed by transcriptional activation and synthesis of ASS protein.[26]

Recently Rabinovich *et al* have confirmed that proliferation of the osteosarcoma cells is supported by down-regulation of ASS, by facilitating pyrimidine synthesis via activation of CAD (carbamoyl-phosphate synthase 2, aspartate transcarbamylase and dihydroorotase) complex.[57] As cytosolic aspartate serves as a substrate for both ASS and for CAD complex,

ASS down-regulation can enhance aspartate availability for CAD for the synthesis of pyrimidine nucleotides to promote proliferation. Thus, aspartate transport can be exploited as an additional therapeutic target in tumors with ASS down-regulation, especially in those ones which develop resistance to arginine-depriving enzymes.

1.3 Tumor resistance towards ADI

ASS-deficient tumors are sensitive to ADI treatment; however, arginine deprivation eventually up-regulates ASS expression in tumor cells and thereby confers resistance towards ADI.[25,58] Transcriptional induction of ASS expression and increase in ASS mRNA level is reported in human embryonic kidney cells and melanoma cells during arginine starvation.[59,60] Transcription factors such as c-Myc and HIF-1α are involved in the up-regulation of ASS expression under arginine depleted conditions.[60] E-box and GC-box are the important sequences located between -85 and -35 nucleotides in the ASS promoter region that modulate ASS expression through their interactions with c-Myc and HIF-1α. Under the normal concentrations of arginine, HIF-1α (but not c-Myc) binds to E-box and thus acts as a negative regulator of ASS expression. Under the conditions of arginine depletion, HIF-1α is degraded and replaced by up-regulated c-Myc, which directly binds to E-box; thus, c-Myc acts as a positive regulator of ASS expression [Fig. 6 of Ref. 60]. Recently reported in melanoma cells, inhibition of ubiquitin-mediated protein degradation is a molecular mechanism responsible for the stabilization and accumulation of c-Myc.[61] Furthermore, various cellular pathways, such as Ras and its downstream ERK/PI3K/AKT kinase cascade are associated with the post-translational modifications of c-Myc, leading to its phosphorylation and stabilization during ADI-PEG20-mediated arginine deprivation conditions. Involvement of Ras/PI3K/ERK signaling pathway in the development of resistance towards ADI treatment suggests that combination of ADI with Ras/ERK, PI3K/AKT inhibitors is a potential therapeutic strategy to improve the anti-cancer response.[62,63]

Development of anti-drug neutralizing antibodies is another possible mechanism of resistance towards ADI-PEG20 treatment.[64] Arginine concentrations were recovered up-to pre-treatment levels in a patient with malignant pleural mesothelioma and in Asian patients with advanced hepatocellular carcinoma following the ADI-PEG20 treatment. This recovery in arginine concentration was found concomitant with an increase in anti–ADI-PEG20 antibody titer.[65] These studies suggest the involvement of drug-associated resistance i.e. anti-drug neutralizing antibodies, rather than tumor-related factors as another possible mechanism of resistance of some tumor cell types towards ADI-PEG20 treatment.[62,63]

1.4 Anti-tumor mechanisms of ADI treatment

1.4.1 Role of autophagy and apoptosis in ADI-mediated arginine deprivation therapy

Due to the involvement of arginine in numerous cellular pathways [Figure 2], the exact anti-proliferative mechanisms of ADI treatment, besides that of arginine depletion, are still elusive. One of the potential pathways involved in the cytostatic and cytotoxic potential of ADI is TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).[66-68] TRAIL plays an important role in the cleavage of Beclin-1 (Atg6) and Atg5 in arginine deprived melanoma cells.[69] Beclin-1 and Atg5 are essential for the formation of autophagosomes and thus crucial for autophagy. Since autophagy serves as a mean to evade apoptosis in arginine depleted cells, TRAIL induced cleavage of Beclin-1 and Atg5 leads to decreased autophagy, thereby increasing apoptosis.[69] Additionally, these two drugs (ADI and TRAIL) complement each other by activating the intrinsic apoptosis pathways. ADI-PEG20 increases cell surface receptors DR4/5 for TRAIL thereby binding TRAIL to these death receptors. As a result, caspase-8 or 10 are activated.[66] ADI-PEG20 treatment also modulates different autophagic pathways involved in the cell survival. AMPK and ERK pathways are activated in ADI-treated prostate cancer cells; while AKT, mTOR and S6K pathways are attenuated. ADI-

PEG20 treatment to CWR22R*v*1 prostate cancer cells induced autophagy, as revealed by the appearance of LC-II only after 30 minutes exposure continues its persistence after 24 hours following ADI-PEG20 treatment.[70,71] Additionally, inhibition of autophagy by chloroquine, a clinically approved anti-malarial agent which inactivates lysosomal functions, accelerates the ADI-induced apoptotic cell death of prostate cancer [70,71] and SCLCs.[39] Thus autophagy has been proposed as a pro-survival mechanism of tumor cells during arginine deprivation.[71]

ADI-mediated arginine deprivation is also known to induce caspase-dependent apoptotic pathways in many of the tumor cells types. ADI-PEG20 treatment activates caspase-3 in *ASS*-methylated malignant lymphoma cells, whereas ASS-positive normal lymphoblastoid cells are resistant to it.[26] Similarly, cell death has been attributed to caspases activation in glioblastoma,[54] melanoma,[38,72] leukemia[73] and pancreatic cancer cells.[74] Moreover, all these studies indicate that inhibition of autophagy leads to further advancement in the ADI-PEG20-mediated demise of tumor cells, suggesting the induction of autophagy as a mechanism of tumor resistance to ADI-PEG20 treatment.

Cumulative pieces of evidence suggest that the activation of caspases is not a sole decisive phenomenon in programmed cell death pathways. Caspase-dependent apoptosis is a major mode of cell death, but in its absence or failure, there are other pathways which can also execute cell death.[75-77] ADI-PEG20 treatment to SCLC, leukemia, retinoblastoma and prostate cancer cells induces apoptotic cell death pathways, however, without activation of caspases, suggesting the role of caspase-independent apoptosis as a cell death pathway.[33,39,69, 70,78] The inter-membrane space of mitochondrion contains proteins such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which can induce apoptotic cell death in a caspase-independent fashion.[79] EndoG is one of the predominant endonucleases that are involved in the regulation of cellular functions such as mitochondrial biogenesis, DNA synthesis and repair. AIF is an FAD-containing flavoprotein which plays an important role in

the stability of an electron transport chain.[80] Nutrient deficiency-mediated stress signals induce mitochondrial outer membrane permeabilization (MOMP), which consequently releases inter-membrane space proteins such as AIF, EndoG and cytochrome *c*. AIF plays a role of central mediator in caspase-independent cell death pathway.[81] AIF, once released into the cytosol, interacts with EndoG and cyclophilin A prior to its translocation into the nucleus.[82] Subsequently after translocation into the nucleus, it triggers cell death either directly, through interaction with DNA, or indirectly, through the production of reactive oxygen species.[73,74,79,80] MOMP promotes both, caspase-dependent and caspase-independent apoptotic pathways, but with different kinetics.[83] Although, the upstream signaling stimulus for both, a caspase-dependent and caspase-independent pathway is the same, *i.e.* via induction of MOMP, their downstream pathways are different. Moreover, nuclear alterations and the changes occurring in mitochondrial trans-membrane potential during caspase-independent pathways are different than those observed in a caspase-dependent apoptotic pathway. [84]

To summarize, growing evidence suggests that autophagy is a prevailing cell survival mechanism in tumor cells undergoing ADI-mediated arginine deprivation. The overall cellular response to ADI-mediated arginine deprivation in different tumor cells operates through a complex cascade, initiating with induction of autophagy and followed by the activation of either caspase-dependent or caspase-independent cell death pathways. It is worth emphasizing that the discrepancy of cellular responses of tumor cells to ADI-mediated arginine depletion in activation of either caspases-dependent or caspases-independent cell death pathways can vary depending on tumor cell type.[38,39,70,71,74] As a result, the precise mechanisms of tumor cell death- consequential of cellular response to ADI-mediated arginine depletion- appear to be complex and variable, and need to be further elucidated.

1.4.2 Inhibition of de novo protein synthesis by ADI-mediated arginine deprivation

Inhibition of *de novo* protein synthesis is another mechanism which can be attributed to the anti-tumor potential of ADI. As extracellular arginine pool is responsible for 40% of *de novo* protein synthesis, ADI treatment to human lung carcinoma cells results in an anti-proliferative effect, mediated by inhibition of protein synthesis.[85] Arginine is present in various compartments such as extracellular, intracellular and citrulline-arginine regeneration *i.e*. cytosolic compartment and it is known to regulate various cellular pathways differently. Protein synthesis mainly utilizes arginine either from the intracellular pool or the citrulline-arginine regeneration mechanism, while polyamines synthesis largely utilizes arginine pool from the intracellular origin.[86,87] Polyamines are synthesized through the methionine salvage pathway via decarboxylation of S-adenosylmethionine (SAM). SAM is a donor metabolite necessary for the transfer of methyl group to DNA and proteins. Human colon cancer (HCT116) cells treated with short hairpin CD44 RNA interference showed a decrease in the total amount of methionine-pool metabolites including polyamines, suggesting the role of polyamines in cancer proliferation.[88]

ADI treatment towards human mammary adenocarcinoma and lung carcinoma cells differently modulates polyamine synthesis and the global protein synthesis. Interestingly, inhibition of protein synthesis has been correlated with the ASS-mediated regeneration of arginine. Cells expressing low levels of ASS (A549) result in decreased protein synthesis (without affecting polyamine synthesis) and those expressing higher ASS levels (MCF-7) are resistant to ADI treatment, as the decreased arginine levels can be replaced by citrulline-arginine regeneration pathway.[85]

1.4.3 Anti-angiogenic effects of ADI-mediated arginine deprivation

As a tumor grows beyond a certain size (2 mm in diameter for most solid tumors), available vasculature within the tumor becomes inadequate to supply sufficient quantities of

essential nutrients for their growth.[89] This results in the generation of hypoxic tumor microenvironment and leads to the development of new blood vessels (angiogenesis) as a colossal requisite of the developing tumors.[90] Accordingly, neovascularization can be stated as one of the decisive phenomena during tumor growth and metastasis.[91] Emerging studies now indicate that not only molecular signals but also metabolic mechanisms regulate angiogenesis.[92] Under stress conditions such as hypoxia, tumor cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF).[93] Increased levels of VEGF activate VEGF receptor 2 (VEGFR2) signaling in the quiescent endothelial cells which in turn initiate angiogenesis.[94-96] Endothelial cells produce 85% of their total amount of ATP via glycolysis. Addiction of endothelial cells on anaerobic rather than aerobic pathway enables them for the formation of vascular sprouts in hypoxic areas.[97,98] Metabolism of tumor endothelial cells resembles that of highly activated endothelial cells because of the tumor induced switch from quiescence to proliferation due to metabolically regulated migration during sprouting.[99,100]

Besides ADI's role in modulation of apoptotic pathways, it has an anti-angiogenic activity that contributes to its anti-tumor potential. The growth, migration and differentiation of human umbilical vein endothelial cells (HUVECs) are strongly impaired in a medium containing recombinant ADI.[101] As a consequence; it results in decreased tube formation with intermittent and incomplete microvascular network. Similarly, Park *et al*. found that *E*. *coli* ADI inhibits angiogenesis by inhibiting tube formation of endothelial cells and neovascularization in Chick Chorioallantoic Membrane (CAM) and Matrigel plug assay.[102]

Suppression of nitric oxide (NO) generation is also another possible mechanism for anti-angiogenic activity of ADI. Since L-arginine is required for nitric oxide synthases (NOSs) to generate NO, the depletion of arginine by ADI suppresses NO synthesis.[102] Potential role of ADI-mediated arginine depletion in inhibition of NO synthesis has been

reported.[103,104] We and others have previously reported that NO promotes tumor growth through the stimulation of angiogenesis[105-107] and regulates cellular interaction by controlling adhesion molecule expression and ultimately cell adhesion.[108,109] NO directly, or indirectly through NO-mediated reactive nitrogen species (RNS), induces the activation of certain angiogenic signaling pathways in the endothelial cells.[110] NO acts as an autocrine mediator in endothelial cell functioning and as a final modulator in VEGF stimulated angiogenesis.[109,111] NO not only mediates angiogenesis but also subsequent vessel maturation[112,113] Moreover, NO is known to inhibit angiostatin and thrombospondin-1, two main inhibitors of angiogenesis.[114] Owing to the important role of NO in angiogenesis, ADI inhibits tumor growth not only by draining the supply of arginine, but also by its anti-angiogenic activity via suppression of NO generation.

To summarize, certain tumor cell types such as, HCCs and metastatic melanomas are invariably deficient in ASS expression and can be specifically targeted by ADI-mediated ADT. It is worth noting that more than one pathway may be attributed to the cytotoxic potential of ADI-mediated ADT [Figure 5]. The anti-tumor potential of ADI may not only be simply accredited to its action as arginine degrading enzyme but also to several other mechanisms important in the cellular functioning of tumor cells. Induction of apoptotic pathways, inhibition of angiogenesis and inhibition of *de novo* protein synthesis are the important mechanisms attributed to the cytotoxic potential of ADI. Moreover, studies have revealed the ADI-mediated modulations in tumor cell-cycle. The fundamental difference of cell cycle modulations in normal and malignant cells should be exploitable as a means of selective demise of tumor cells and ADI, in combination with other anti-cancer chemotherapeutic agents, which can be a potential strategy to improve chemo-sensitization against tumor cells.[115-118]

2. Arginase

Arginase (E.C.3.5.3.1) is a mammalian enzyme which catalyzes the conversion of arginine to ornithine and urea. Arginase is considered as an enzyme responsible for the cyclic nature of urea cycle, since only the organisms containing arginase are able to carry out the complete urea cycle.[119] Two distinct isoforms of mammalian arginase have been identified which are encoded by two separate genes.[120] Type I arginase (arginase I) is located in the cytosol and is mainly expressed in liver. Type II arginase is located in the mitochondrial matrix and is expressed in extra-hepatic tissues.[121,122] Intracellular regulation of arginase expression is of immense importance as it has crucial implications for the synthesis of essential cellular metabolites,[123] For example, cytosolic co-localization of arginase I with ornithine decarboxylase (ODC) preferentially utilizes ornithine for the biosynthesis of polyamine. On the other hand, due to its co-localization with ornithine aminotransferase (OAT) in the mitochondria, arginase II directs ornithine for the production of proline and glutamine.[124,125]

2.1 PEGylated recombinant human arginase I

Elevated requirements of arginine by tumor cells were first identified in 1947 and preferential utilization of arginine by tumor bearing animals was revealed in 1953.[126,127] The use of bovine and murine arginase in arginine deprivation therapy was prevailing until the advent of recombinant DNA technology,[128-130] followed by the pervasive use of recombinant human arginase in subsequent decades.[131,132] Arginase from bovine and murine sources has been extensively used for the arginine deprivation therapy *in vitro*. However, limited success was achieved *in vivo* due to its alkaline optimum pH and very low affinity for the substrate. Human arginase I also has a serious limitation of very short circulatory half-life (Approx. 30 minutes).

To extend plasma half-life of arginase, PEGylation has been applied successfully. PEGylated recombinant human arginase I (rhArg-Peg_{5000mw}) had efficient catalytic activity at physiological pH with improved *in vivo* half-life of 3 days. Furthermore, rhArg-Peg_{5000mw} was found to have significant tumor inhibitory activity in BALB/c nude mice bearing HCC xenografts.[131] Notably, these results were consistent with those demonstrated by Tsui and co-workers.[133] Recently, a bio-engineered form of human arginase I was developed by the co-factor replacement, the replacement of two Mn^{2+} ions by Co^{2+} ions. The modified Co^{2+}-arginase I resulted in 10-fold increase in the catalytic activity and 5-fold greater stability at the physiological pH. Nevertheless, IC_{50} values for killing human HCC and melanoma cell lines were lowered by 12-15 folds.[134] More recently, modifications in bioengineered Co^{2+}-arginase I were performed by conjugating 5-kDa PEG to enhance plasma half-life. This modified version of bioengineered arginase I (Co-hArgI–PEG) was proven to be cytotoxic by significantly increasing the expression of caspases-3 in HCC and pancreatic carcinoma (PC) tumor xenografts.[135] Lately, the cytotoxic potential of Co-hArgI–PEG was identified in acute myeloid leukemia (AML) and glioblastoma cells. AML cell lines were found sensitive towards Co-hArgI–PEG-mediated arginine deprivation with very low (58-722 PM) IC_{50} values, suggesting a very high potential of Co-hArgI–PEG-mediated arginine depletion in AML cells.[136] Moreover, Co-hArgI–PEG-mediated arginine deprivation has been demonstrated to induce caspase-independent, non-apoptotic cell death in human glioblastoma cells.[137] Alternative method to extend the plasma half-life of recombinant human arginase also has been established. Plasma half-life of a fusion protein form of a recombinant human arginase (rhArg-Fc, constructed by linking rhArg to the Fc region of human immunoglobulin IgG1), was evidenced to significantly extend up-to approx. 4 days.[138] In addition, rhArg-Fc was confirmed to conspicuously inhibit the cell growth of human HCC cells *in vitro* and *in vivo*.[138]

Last decade has evidenced a prevalent use of recombinant human arginase-mediated ADT in numerous cancer cell types, mainly metastatic HCC and melanomas.[131,139,140] Currently, PEGylated derivative of recombinant human arginase I is undergoing clinical trials for the treatment of human HCC.[141,142] Moreover, initiatives are now being taken to overcome the possible problem of accumulation of PEGylated products in the liver by impending approaches such as fusion proteins.[138]

2.2 Anti-tumor mechanisms of arginase-mediated arginine deprivation

Selective starvation of L-arginine in tumor cells, which are auxotrophic for L-arginine, is one of the most important anti-tumor mechanisms of ADT. Arginase can render its cytostatic effect as a result of modulations in the cell cycle proteins, whereas, cytotoxic effects rendered by arginase I-mediated arginine deprivation have been proposed as a result of induction of potential cell death pathways namely apoptosis and probably by 'autophagic cell death'. Summarized below are the current understandings of the molecular mechanisms of cytostatic and cytotoxic effects rendered by arginase-mediated ADT.

2.2.1 Role of autophagy in arginase-mediated arginine deprivation

Autophagy is a key sensing and regulatory mechanism of cells in nutrient deprived conditions. Under stress conditions, autophagy functions as a bio-energy management system by recycling cell organelles and damaged and/or long-lived proteins.[143] Although autophagy seems to be a survival mechanism of the cells, there is a growing evidence of accumulation of autophagosomes and other autophagic markers in dying cells unable to process apoptosis, raising the term 'autophagic cell death'.[144-147] However, the term 'autophagic cell death' is based on morphological features rather than the causative role of autophagy in cell death. New definition of 'autophagic cell death' has been proposed,

implying that cell death must occur without the involvement of apoptotic machinery, (caspase activation) but with an increase in autophagic flux.[148,149]

Mammalian target of rapamycin (mTOR) is a key regulator of coupling cell growth and nutritional status of the cell.[150,151] Autophagy is induced by the inhibition of mTOR signaling pathway.[152] During nutrient affluent conditions, mTOR is involved in the negative regulation of Atg1 (autophagy related gene 1) which inhibits autophagy.[153,154] Arginase-mediated arginine deprivation leads to decreased levels of ATP, which in turn activates the adenosine 5′-monophosphate-activated protein kinase (AMPK). Activated AMPK eventually inhibits the mTOR-signaling pathway, manifested by the reduced phosphorylation of key downstream molecules, such as 4E-BP1 (Eukaryotic translation initiation factor 4E-binding protein-1). Dephosphorylation of 4E-BP1 is observed in Chinese hamster ovary (CHO), human melanoma cells and human prostate cancer cells following their exposure to recombinant human arginase I.[65,155,156] Phagosome/lysosome activity is also significantly increased following an incubation of human tumor cells in L-arginine deficient medium.[157] Additionally, studies carried out by Hsueh *et al.*[156] evidenced no significant induction of apoptotic mechanisms in prostate cells after their exposure to rhArgI, suggesting the role of autophagic cell death, rather than apoptosis, as an alternative cell death mechanism. In addition, autophagy has often accompanied damaged mitochondria and higher levels of reactive oxygen species (ROS).[158,159] Acute generation of ROS has been attributed to causing severe damages to the cellular macromolecules, which in consequence, leads to necrosis of the tumor cells.[160,161] Overall, arginase leads to deprivation of arginine, in consequence, it inhibits mTOR pathway during the deprivation and thus forcing tumor cells to undergo ‘autophagic cell death’ pathway.[162]

SLC38A9, a member 9 of the solute carrier family 38, has been recently identified as an integral component of the lysosomal machinery that controls amino acid-induced mTOR

activation.[163,164] Amino acid starvation in human embryonic kidney (HEK293T) cells with stable expression of SLC38A9 has been shown to activate mTOR in a sustained manner. Moreover, shRNA-mediated silencing of SLC38A9 results in a reduction of arginine-induced mTOR activation. Also, depletion of SLC38A9 impaired mTOR activation induced by cycloheximide (a protein synthesis inhibitor which induces accumulation of intracellular amino acids), further suggests the role of SLC38A9 in mTOR activation at the lysosomal rather than at the plasma membrane. These studies have demonstrated that SLC38A9 acts as an upstream positive regulator tor mTOR functioning and thereby modulating autophagy in arginine-deprived tumor cells.

Although some studies have advocated autophagy as a cell death mechanism of arginase-mediated ADT,[156,157] many groups have explained it as a pro-survival mechanism; mainly by postponing the activation of apoptosis.[38,161] Thus, understanding the exact role of autophagy in arginase-mediated cell death pathways is a complicated episode.[162,165] Therefore, much need to be elucidated about these new findings related to 'autophagic cell death' and caution must be taken to assign autophagy as a cell death pathway in arginase-mediated ADT.

2.2.2 Role of apoptosis in arginase-mediated arginine deprivation

The role of autophagy, either in cell survival or in cell death, depends on many factors such as cell type, nature and severity of the stimuli and so on.[166] If the attempt of the cells to survive through autophagy fails, apoptotic pathways take over and ultimately cause cell death.[143] Inhibition of autophagy in amino acid deficient conditions induces tumor cell death, mainly because of further exacerbation of energy dearth.[167,168] Also, longer persistence of autophagy is proposed to eventually lead the activation of caspase-dependent cell death pathways, as autophagy and apoptotic cell death pathways are interconnected and

also share some common pathways through the induction of the membrane permeability transitions.[169-171] Induction of apoptotic pathways is another consequence of arginine depletion and anti-tumor mechanism of arginase I-mediated arginine deprivation.

Involvement of apoptosis as a cell death mechanism in arginase-mediated ADT has been illustrated in various literature reports. Annexin V is known to selectively stain the cells, which are destined for apoptosis or in the process of apoptosis. 33% of human melanoma cell population was destined for apoptotic cell death following rhArg treatment.[139] Arginase I-mediated arginine deprivation led to the transcriptional up-regulation of *caspase 3*, the intrinsic mitochondrial pathway of apoptosis, which is marked by the change in mitochondrial membrane potential.[172] Recently, an anti-leukemic potential of PEGylated-arginase has been attributed to kinases general control nonderepressible 2 (GCN2)-mediated induction of apoptosis in T-ALL cells.[173]

2.2.3 Cell cycle arrest by arginase-mediated arginine deprivation and combination approaches

rhArg-Peg_{5000mw}-mediated arginine deprivation in various HCC cells results in their cell cycle arrest at G_2/M phase, by decreased expression levels of cyclin B1 and cdc2, or in S phase, by a transcriptional up-regulation of cyclin A1 [Ref. 140]. rhArg-Peg_{5000mw}-mediated arginine depletion was witnessed to impair the expression of cyclin D3 in T-ALL cells, which was followed by an arrest of the cells in the G_0-G_1 phase of the cell cycle and induction of apoptosis.[172] Recent investigations of rhArg-Fc-mediated arginine deprivation in human HCC cells exhibited cell cycle arrest at S phase.[138] The exact mechanisms of these findings are still elusive, but the possible reasons seem to be the increased expression of cyclin A and declined transcription levels of p27 and p21 (the key cyclin kinase inhibitors).

Owing to the evidence of cell cycle arrest, a combination of arginase and other cell-cycle specific anti-cancer chemotherapeutics as potential anti-tumor approaches have been established. Synergistic effects of rhArg-Peg$_{5000mw}$ with 5-fluorouracil (5-FU, uracil analog which interferes with RNA and DNA synthesis) and cytarabine (Ara-C, anti-metabolic chemotherapeutic agent) have been investigated on the inhibition of proliferation of HCC and T-ALL cells, respectively.[131,172] Treatment of either rhArg-Peg$_{5000mw}$ or Ara-C alone induces a heterogeneous anti-tumor effect *in vivo*, whereas, combined treatment of rhArg-Peg$_{5000mw}$ and Ara-C induces a homogenous prevention of spleen growth, leading to the prolonged survival in all of the T-ALL bearing mice.[172] Moreover, combined treatment of PEGylated recombinant human arginase I and oxaliplatin has been demonstrated to synergize the inhibiting effect on tumor growth and enhanced overall survival probability as compared to PEGylated recombinant human arginase I or oxaliplatin treatment alone.[174]

Altogether, arginase has an advantage over ADI that it is efficacious in both ASS-negative and OTC- negative tumors,[59] whereas ADI is efficacious only in ASS-negative tumors. The tumor cell types expressing ASS are resistant to arginine deprivation treatment by ADI.[25,26,54,61,131] Even though arginase has been considered as a potential drug candidate over a period of six decades, low substrate specificity (high k_m of 2-4 mM), short plasma life and optimum alkaline pH (pH 9.3) limit *in vivo* applications of arginase.[131,140] In addition, robust homeostatic mechanisms in the body allow faster restoration of plasma free arginine, making *in vivo* arginine deprivation by arginase more difficult. Most of the scientific efforts nowadays pay attention to these limiting characteristics of arginase.[134,175,176]

3. Arginine decarboxylase

Arginine decarboxylase (ADC) (E.C. 4.1.1.19) metabolizes arginine to agmatine, one of the minor metabolic products of arginine. ADC is mainly found in plants, bacteria and mammalian liver and brain membranes.[177,178] The mammalian ADC is different from other

sources and distinct but related to ODC.[179] Although, arginine decarboxylation by ADC is a minor metabolic route, its product i.e. agmatine has a significant role in numerous cellular pathways.[180] Agmatine modulates the polyamine metabolism through its negative interaction with ODC.[181] Agmatine also confers an inhibitory effect on intracellular polyamine content by inhibiting polyamine uptake[182] and probably by increased polyamine catabolism.[183] Mayeur *et al*.,[184] has reported the effect of agmatine accumulation on polyamine metabolism, cell proliferation and cell cycle distribution in human colon adenocarcinoma epithelial cell lines. Due to the agmatine-mediated reduction in polyamine synthetic capacity of the cells, agmatine markedly inhibits the cell proliferation of HT-29 and Caco-2 cells in a dose dependent manner, without affecting cell membrane integrity. Moreover, agmatine modulates the cell cycle progression by decreasing ODC activity and expression.[181,185] As ODC plays an important role in the G_1/S progression of the cells, agmatine-mediated modulations in ODC expression lead to modifications in the cell cycle progression.[186] Additionally, agmatine also has been shown to delay the expression of cyclins in tumor cells, leading to the modifications in the cell cycle progression.[184]

ADC has been investigated for the enzymatic degradation of arginine in normal and malignant cell cultures.[187] Arginine deprivation in human diploid fibroblasts (normal cells), achieved using human recombinant ADC, resulted in the cell cycle arrest at G_1/G_0. While treatment of 0.1 unit ml^{-1} ADC to HeLa (Human cervical cancer) cells resulted in cell cycle arrest with an initiation of cell death after 2 days.[187] Similar results were evidenced in the studies by Wheatley *et al*.,[188] where 5 units ml^{-1} ADC was found as effective as arginase in the inhibition of HeLa cells and cell cycle arrest at G1 (quiescence) in fibroblasts.

Although some research groups have exhibited ADC as a potential anti-tumor enzyme, only a few reports are available to support this fact [Table 1].[187,188] Even though ADC possesses low *K*m and can degrade arginine very rapidly, the serious problem is related

to its product i.e. agmatine. Agmatine is toxic to normal cells when its concentration reaches a millimolar level, particularly when free arginine levels are low. Additionally, agmatine is not converted back to arginine under normal physiological conditions, which may lead to its accumulation and toxicity to normal cells.[189] Though recombinant human ADC expressed in *E. coli* has been evidenced more active than Sigma enzymes prepared from other sources, its PEGylation has been shown to result in the loss of its entire activity.[187,189] To consider the further rational use of this prospective enzyme as potential anti-cancer modality, it clearly warrants further evaluation [Table 3].

Concluding remarks

Sufficient evidence has been accumulated indicating that arginine catabolic enzymes-based approaches may be an effective way to target malignant cells. These enzymes control tumor cell proliferation as well as make them highly vulnerable to cell-cycle specific chemotherapeutic agents. This combinatorial approach is one of the potential strategies to maximize the efficacy to obliterate the tumor cells. Extensive research of the arginine metabolic pathways led to the establishment of arginine-depriving enzymes as a potential anti-cancer strategy against arginine auxotrophic tumors. However, many of these enzymes can be co-expressed in the cells, which results in complex interactions. For example, arginine is a common substrate for arginase as well as NOS. The specific role of NO, either in inhibition or induction of cell proliferation is dependent on numerous factors like its interaction with other free radicals, cellular makeup, tumor milieu, proteins present the cellular microenvironment and also upon the chemical and biological heterogeneity of NO. NO has been known to demonstrate bipolar cellular effects and often termed as "*double-edged sword*". Although, NOS remains a viable candidate for cancer treatment, the precise role of NO in the tumor microenvironment is extremely complex and conflicting. Also, the preferential utilization of arginine by arginase and/or NOS pathway is not fully understood.

Thus, many of these pathways warrant further research to understand the arginine metabolism at cellular and molecular levels involving upstream and downstream pathways of the enzymes involved.

It should be noted that modulation of the immunological responses is one of the major roles of arginine availability. Arginine metabolism in myeloid-derived suppressor cells via arginase and/or NOS markedly impairs the T-cell responses that would eradicate and remove tumor cells.[190] Many excellent articles are available which focus on the role of arginine in immunological aspects of the tumors. [191-194] It would suffice to say here that the arginine deprivation therapy may have further anti-tumor effect through restoration of anti-tumor immunity.

Arginine dependence of the tumor cells has been considered as the "*Achilles heel*" of tumor cells.[195] Inability of tumor cells to proliferate in the absence of arginine can be targeted for their selective destruction by arginine depriving enzymes. Large numbers of enzyme-based anti-cancer therapies are currently undergoing clinical evaluation. It is encouraging that arginase and arginine deiminase already have achieved considerable success, without causing detrimental side effects and with high tolerability.[51,63,141] The knowledge acquired about the PEGylation has helped in the generation of adducts of potential value, overcoming the serious limitations of the anti-cancer enzymes of the non-human origin. The approach of enzyme-mediated arginine deprivation therapy is highly challenging, however rewarding upon success due to the provision of overturning the cancer dogma.

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Figure legends

Figure 1: **Amino acid response (AAR) pathway**

Restriction of essential amino acids activates the general control nondepressible protein 2 (GCN2) kinase by increasing uncharged t-RNA pool.[196] Activated GCN2 kinase phosphorylates the translation initiation factor eIF2α. Phosphorylated eIF2α binds more tightly to eIF2β, inhibiting the exchange of GDP for GTP. Inhibition of GDP exchange for GTP further inhibits the binding of eIF2 complex to methionine aminoacyl tRNA, leading to inhibition of translational initiation.[197] Recently, SLC38A9 has been identified as an

upstream positive regulator of the mTOR pathway. Amino acids activate the RAG GTPases, which then recruit mTOR to the lysosomal surface. Rheb also localizes to lysosomal membrane. mTOR activation occurs only when both RAG GTPases and Rheb are active. Upon amino acid deprivation, tuberous sclerosis complex (TSC) translocates to lysosomal surface and promotes GTP hydrolysis by Rheb and thereby inhibiting mTOR complex.[164]

Figure 2: Involvement of arginine in human physiology

Arginine is a dibasic, cationic amino acid and is considered as 'conditionally essential' amino acid. Arginine plays a crucial role in innate and adaptive immunity. For example, increased role of arginine in myeloid-derived suppressor cells results in the impairment of T-cell proliferation and function.[190] Arginine has been identified as the sole physiological precursor for nitric oxide (NO), a key performer in many cellular regulatory functions. Arginine also is a precursor of two important amino acids, proline and glutamate.[198] One of the most important roles of arginine is its implication in the synthesis of polyamines through the diversion from NO synthesis pathway. Polyamines are known to promote tumor growth, invasion and metastasis.[199] Arginine also plays a vital role in the synthesis of nucleotides, creatine, agmatine and hormones such as insulin and prolactin.[200]

Figure 3: Arginine synthesis and homeostasis pathways

Arginine is synthesized as an intermediate in the urea cycle. Arginine homeostasis is mainly achieved by catabolism. In neonates, the gene expression of arginine anabolic enzymes such as 1-pyrroline-5-carboxylase, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) is low. Thus, arginine is considered as an essential amino acid in neonates. After birth, the expression of ASS and ASL increases and expression of arginase is found undetectable at this stage.[201] Arginine can be degraded by arginase, ADC, ADI and NOSs (Please note that ADI is not a mammalian enzyme). The products of arginine catabolism play important roles in tumor cell biology. For example, ornithine, the product of arginase, is diverted to polyamine synthesis via ornithine decarboxylase. NOSs degrade arginine into citrulline and NO. Citrulline is recycled to urea cycle, while NO is as a modulator of important metabolic and signaling cascades. Agmatine is synthesized by decarboxylation of arginine via ADC and plays an important role in neurotransmission.

Figure 4: Timeline of important advancement in arginine deprivation therapy of cancer

Figure 5: Schematic representation of cytostatic and cytotoxic pathways involved in arginine deprivation therapy

Arginine deprivation therapy (ADT) can potentially modulate numerous cellular and signaling pathways rendering their cytotoxic and cytostatic pathways. Induction of apoptotic pathways, inhibition of angiogenesis and inhibition of *de novo* protein synthesis are the important mechanisms attributed to the cytotoxic potential of ADT. Moreover, ADT-mediated modulations in tumor cell-cycle can be exploited as a means of tumor growth arrest.

List of Figures

Figure 1:

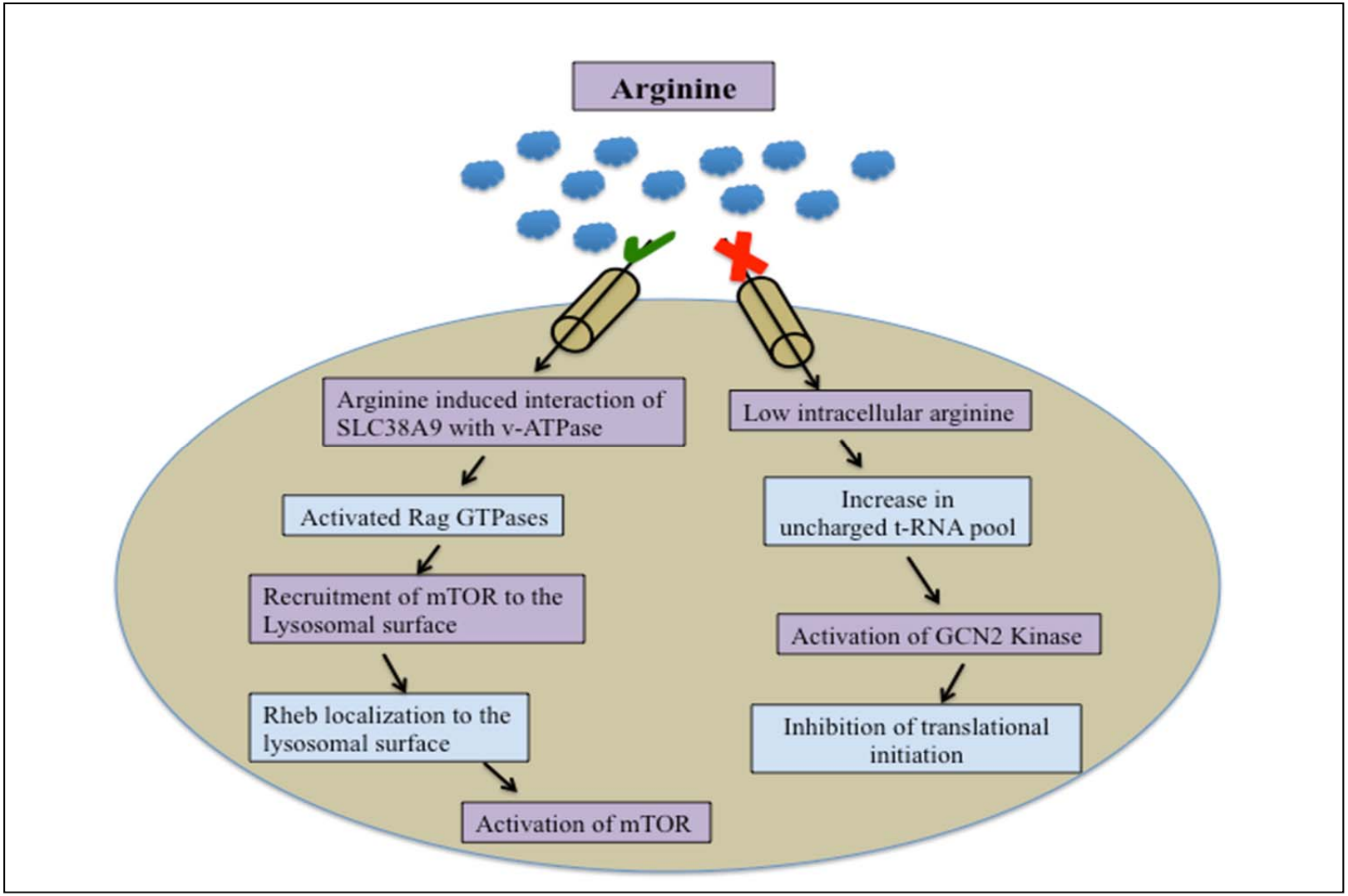

Figure 2

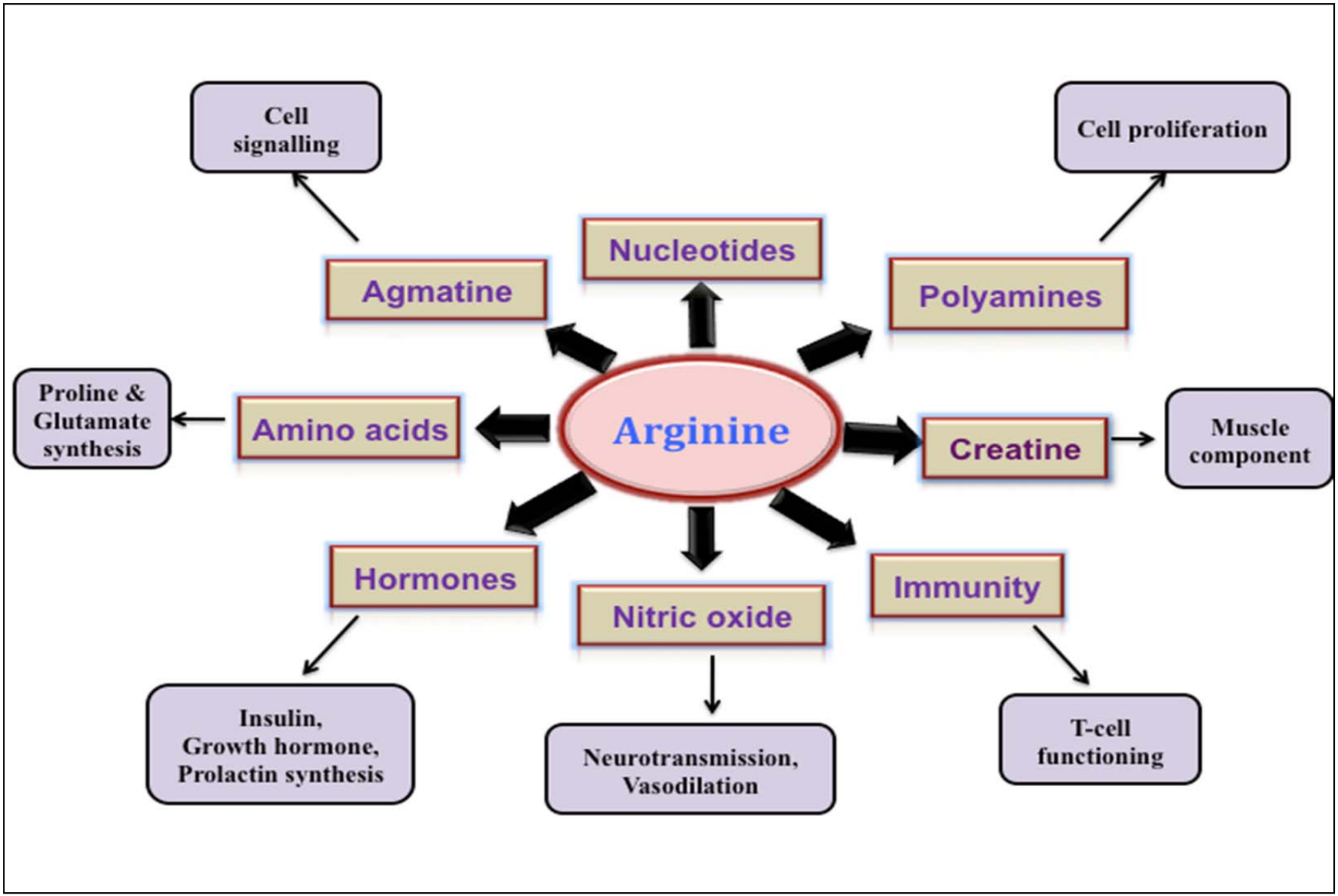
Cell signalling
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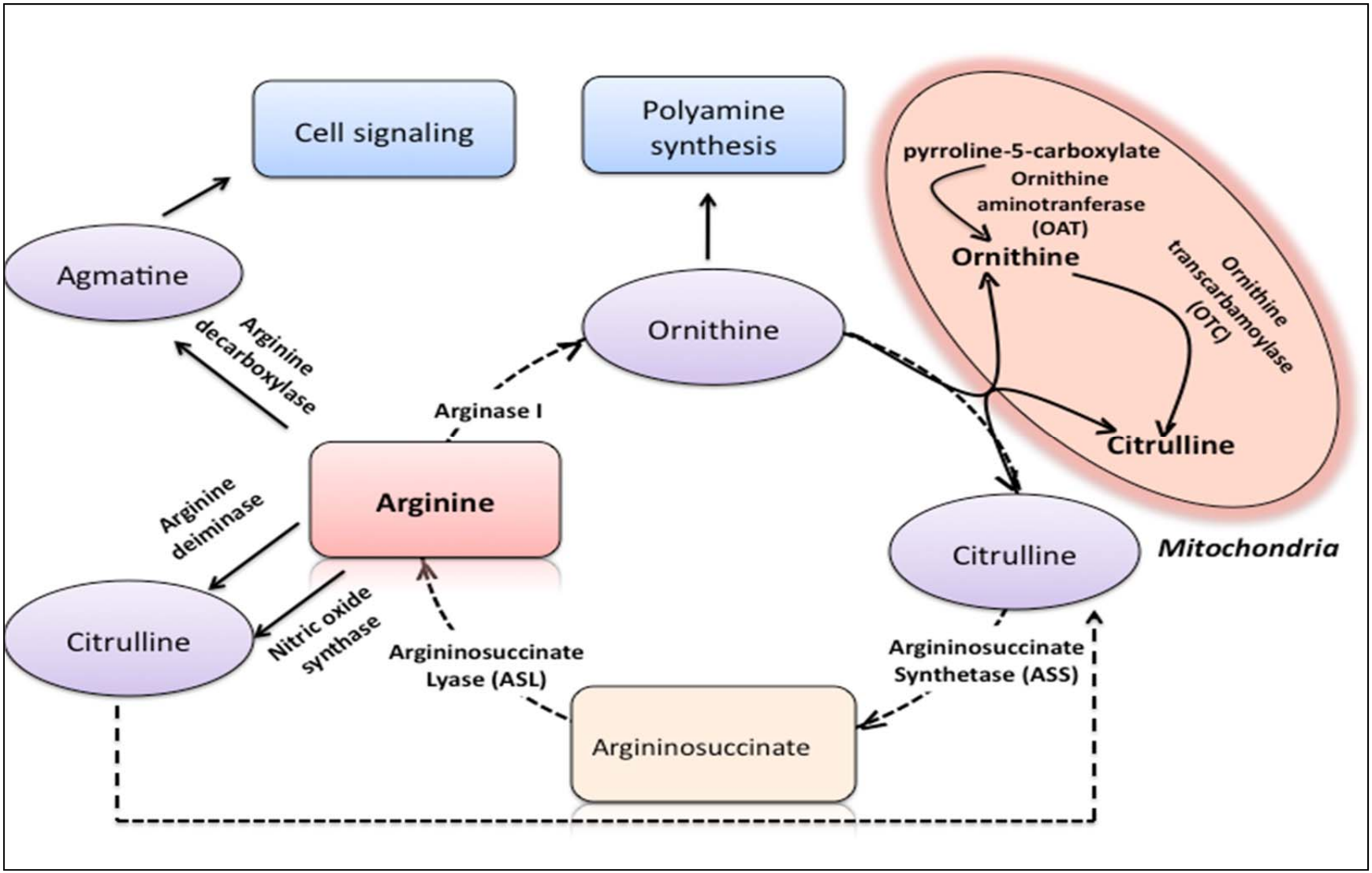
Figure 3


Cell signaling
Polyamine synthesis
pyrroline-5-carboxylate
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Argininosuccinate

Figure 4

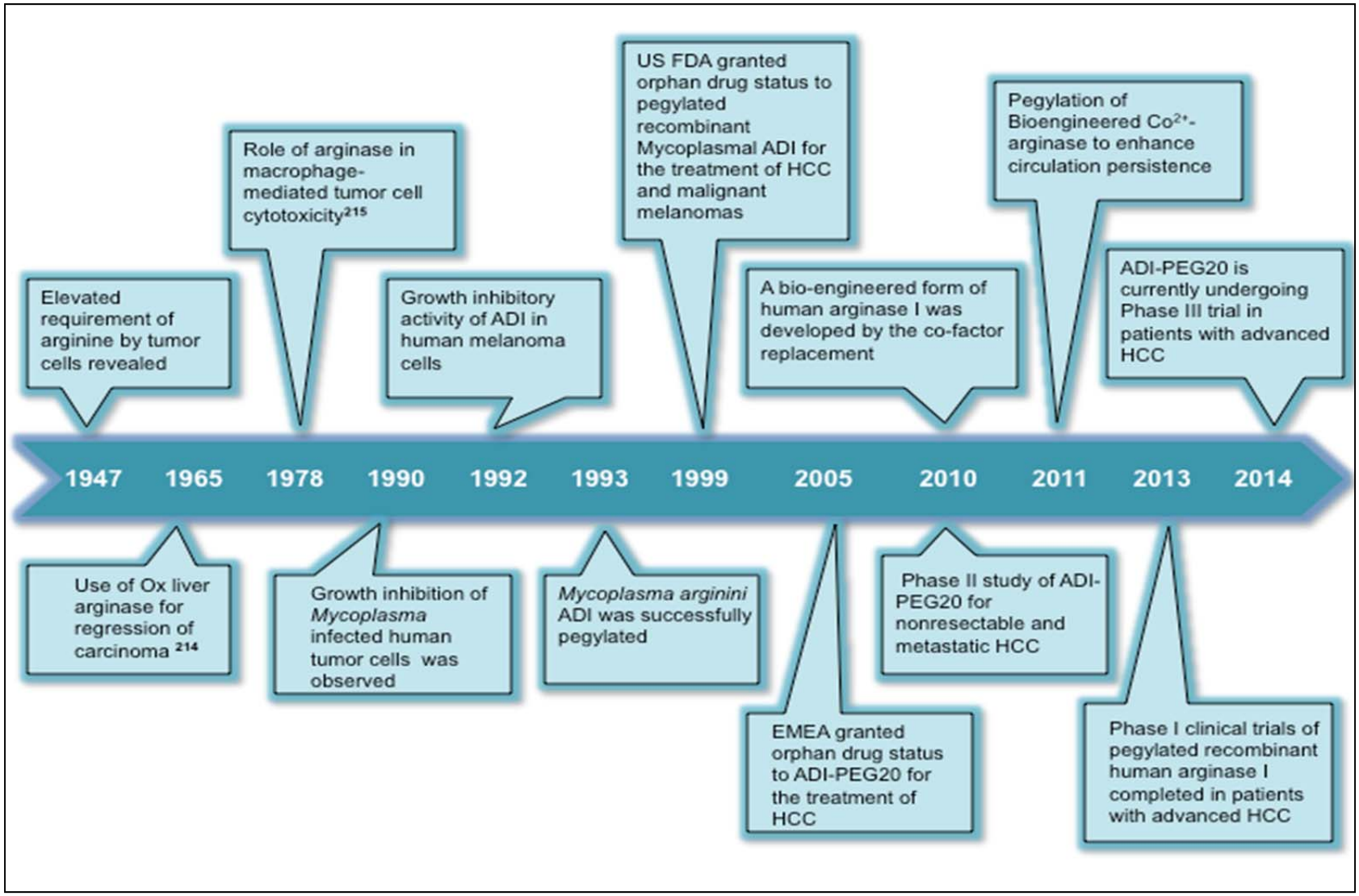
1947
Elevated requirement of arginine by tumor cells revealed
1965
Use of Ox liver arginase for regression of carcinoma [214]
1978
Role of arginase in macrophage-mediated tumor cell cytotoxicity[215]
Growth inhibition of *Mycoplasma* infected human tumor cells was observed
1990
1992
Growth inhibitory activity of ADI in human melanoma cells
1993
Mycoplasma arginini ADI was successfully pegylated
1999
US FDA granted orphan drug status to pegylated recombinant Mycoplasmal ADI for the treatment of HCC and malignant melanomas
2005
EMEA granted orphan drug status to ADI-PEG20 for the treatment of HCC
2010
A bio-engineered form of human arginase I was developed by the co-factor replacement
Phase II study of ADI-PEG20 for nonresectable and metastatic HCC
2011
Pegylation of Bioengineered Co^{2+}-arginase to enhance circulation persistence
2013
Phase I clinical trials of pegylated recombinant human arginase I completed in patients with advanced HCC
2014
ADI-PEG20 is currently undergoing Phase III trial in patients with advanced HCC

Figure 5

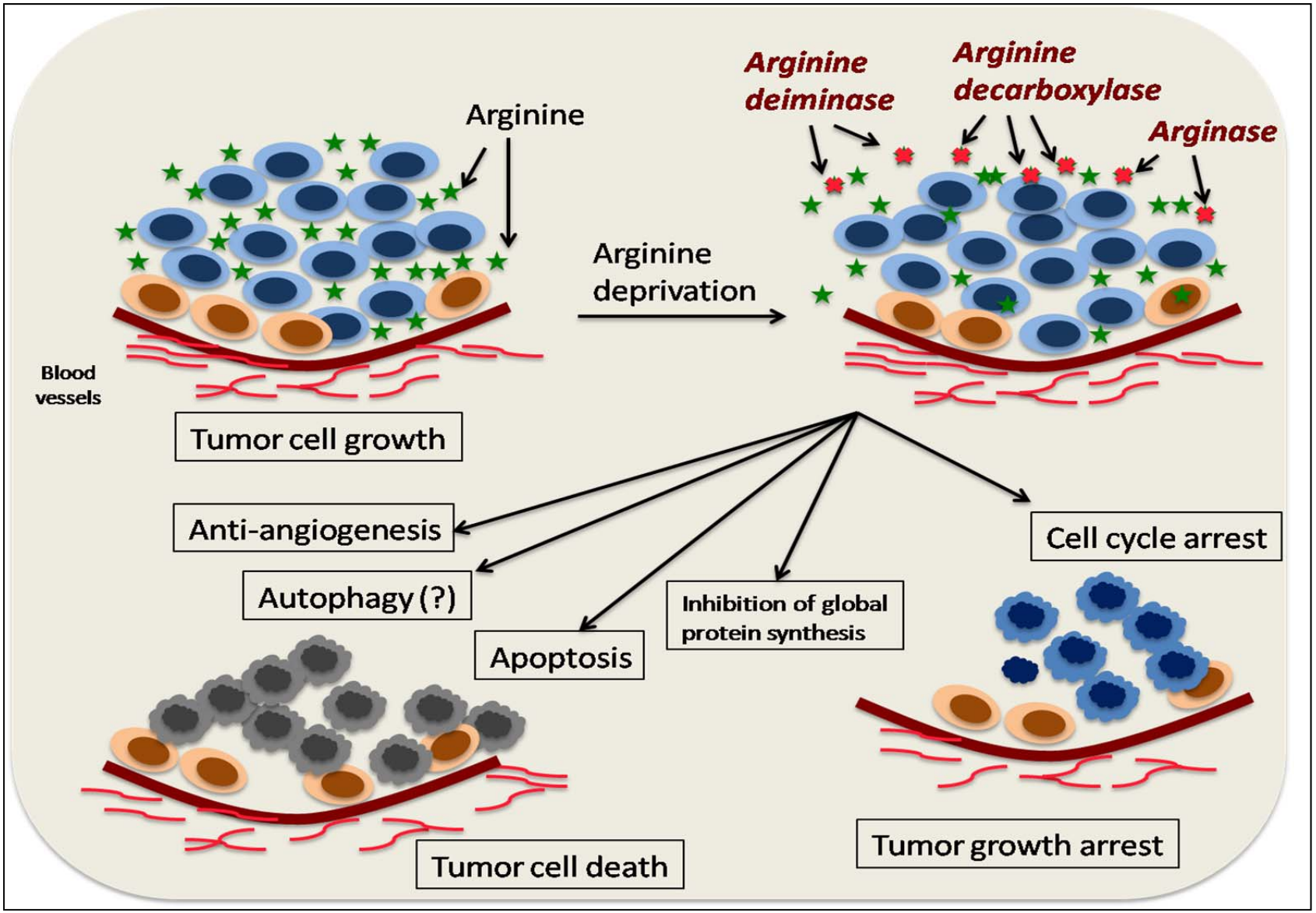
Arginine
Arginine deiminase
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Table 1: Use of arginine catabolizing enzymes in ADT (Experimental studies) [* indicates Tumor xenograft experiments]

Enzyme used for deprivation	Cell line	Source and Cell type	Studies carried out	Reference
ADI	HSC-3 HSC-4	Human tongue squamous carcinoma	Cell growth inhibitory effect of ADI (purified from *Mycoplasma* infected cell lines) in comparison with arginase	[43]
	CaSki	Human cervix squamous		
	C41	Human carcinoma		
	A549	Human cervix squamous epithelium		
	SCC	Human colon adenocarcinoma		
	T98G	Human glioblastoma		
	HeLa	Human cervix	Concentration dependent effect of ADI on cell proliferation	[102]
	CHO	Chinese hamster ovary		
	FF9	Fetal foreskin fibroblast		
	HUVEC	Human umbilical vein endothelium	Anti-angiogenesis effect of ADI by inhibiting capillary-like tube formation	
	SNU-1	Human stomach adenocarcinoma	Anti-proliferative effect and ADI induced cell cycle arrest and apoptosis	[202]
	L5178Y	Mouse lymphoblastic leukemia	Inhibition of cell division	[203]
	MCF7	Human mammary adenocarcinoma	Effect of ADI on the regulation of cellular protein and polyamine synthesis	[85]
	A549	Human lung carcinoma		
	SNUOT-Rb1	Human retinoblastoma	ASS expression related sensitivity of cells towards ADI	[204]
	Y79	Human retinoblastoma		
ADI-PEG20	CWR22Rv1*	Human prostate	Autophagy and caspase independent apoptosis	[71]
	A2058	Human melanoma	Combination effect of ADI and TRAIL,	[66]
	SK-Mel-2	Human melanoma		
	HUVE	Human umbilical vein endothelium	Cell cycle progression and apoptotis	[78]
	SaOS	Human osteosarcoma	Inhibition of NO using Pegylated ADI	
	WAC2	Human neuroblastoma		
	Y-79	Human retinoblastoma		
	Meth AC14	Human sarcoma	Effect of ADI-PEG20-mediated deprivation on the production of NO	[103]
	SK-LC-13*	Human small cell lung		
	SW1271	Human small cell lung	ASS expression related sensitivity of cells towards PEG-ADI, Induction of autophagy and caspase-independent	[39]

	NCI-H82	Human small cell lung	apoptosis	
	A375 SK-mel-2* SK-mel-28* SK-hep 2* SK-hep 3* HEP3B	Human melanoma Human melanoma Human melanoma Human HCC Human HCC Human HCC	Specificity of ADI for degradation of arginine and other amino acids; ASS expression dependent sensitivity of HCC and melanomas towards ADI	[47]
	A2058* SK-MEL-2 MDA-MB-231	Human melanoma Human melanoma Human breast	Involvement of Ras/PI3K/ERK pathway in induction of c-Myc stabilization and up-regulation of ASS	[61]
	Karpas-422 MyLa SeaX	Human B-cell lymphoma Human T-cell lymphoma Human T-cell lymphoma	Correlation between *ASS* methylation status and sensitivity of the cells towards ADI	[26]
	OEC-M1 SCC-15 HONE-1	Human head and neck cancer Human head and neck cancer Human head and neck cancer	Potential clinical correlation between ASS expression and tumor prognosis	[205]
	A375 Sk-Mel2 A2058 MEL-1220	Human melanoma Human melanoma Human melanoma Human melanoma	The role of ASS gene expression in ADI response/resistance	[72]
	MIA-PaCa-2* PANC-1 Capan-1 HPAF II	Human pancreatic cancer Human pancreatic cancer Human pancreatic cancer Human pancreatic cancer	The role of ASS gene expression in ADI response/resistance	[74]
Bovine liver arginase	L1210 HeLa A375 MEWO SAos-2	Murine lymphocytic leukemia Human cervical adenocarcinoma Human melanoma Human melanoma Human osteogenic sarcoma	Cell proliferation and non- recoverable cell death of malignant cells on restoration of arginine Cell proliferation and ASS expression dependent recycling of citrulline to arginine	[187] [132]
rh-Arginase I	IPEC-1	Pig intestinal porcine epithelial cells-1	LPS- induced cell damage involving mTOR and TLR4 pathways	[206]

rhArginase I-PEG_{5000mw}	PC-3 DU-145 LNCaP	Human prostate Human prostate Human prostate	Expression levels of ASS and OCT, rhArginase I- mediated modulations in mTOR signaling pathway	[156]
	A375* SK-MEL-2 SK-MEL-28	Human melanoma Human melanoma Human melanoma	Proliferation and cell cycle progression of melanoma cells, modulations in the cell cycle and apoptosis-related genes	[139]
	B16-F0* L1210 HeLa	Mouse melanoma Murine lymphocytic leukemia Human cervical adenocarcinoma	Rescue of the arginase treated cells by norvaline (Arginase inhibitor)	[207]
	Hep-3B* SK-HEP-1 Huh7 SK-MEL-28 PLC/PRF/5	Human HCC Human liver adenocarcinoma Human HCC Human melanoma Human primary hepatoma	Gene expression profiling of *ASS* and *OTC*, Synergistic effect of pegylated rhArginase I with 5-Fluorouracil on cell growth inhibition	[131]
	CCRF-CEM* Jurkat Molt-3	Human T-ALL Human T-ALL Human T-ALL	Combination effect of pegylated rhArginase I with Cytarabine (Ara-C) on expression of cyclins	[172]
	HepG2* Hep3B*	Human HCC Human HCC	Effect of pegylation of rh-arginase I on its anti-tumor efficacy, immunogenicity and circulation half life	[133]
	HepG2* PLC/PRF/5* Hep3B	Human HCC Human HCC Human HCC	Cell cycle progression and transcriptional modulation of cyclins and/or CDKs	[140]
	CCRF-CEM* Molt-4 H9 Lousy Jurkat HPB-ALL KOPTK1	Human T-ALL Human T-ALL Human T-ALL Human T-ALL Human T-ALL Human T-ALL Human T-ALL	Global arrest in protein synthesis; Central role of phospho-eIF2a signaling and the kinases (GCN2 and PERK) in the induction of T-ALL cell apoptosis by rhArginase I-PEG_{5000mw}	[173]
Bioengineered human arginase I	HepG2* Panc-1*	Human HCC Human pancreatic carcinoma	Effect of Co^{2+} substitution of the Mn^{2+} on catalytic activity and stability of human arginase I	[135]
	Hep3b A375	Human HCC Human melanoma	Effect of Co^{2+} substitution of the Mn^{2+} on cytotoxicity	[134]

Table 2: Clinical investigations involving arginine depriving enzymes

Enzyme	Cancer type	Phase of a clinical trial	Number of patients	Clinical outcomes	Common side effects	Post-treatment levels of plasma arginine[#]	Reference
ADI-PEG20	HCC	II	71	SD:31% (22/71) DCR: 31% (22/71)	Hypersensitivity/skin rash, local tissue reaction at injection site, hyperuricemia, pruritus, fatigue, hyperammonemia, fever, diarrhea	< 2 µM	[63]
	ASS (-) melanoma	I	17	PR: 23.5 % (4/17) SD: 29.4 % (5/17) CBR: 52.9 % (9/17)	Mild/moderate discomfort at the intramuscular injection site, neutropenia and thrombocytopenia, anaemia, fatigue	Undetectable	[25]
	HCC	I/II	19	CR: 11% (2/19) PR: 37 % (7/19) SD: 37% (7/19)	Occasional elevation in serum lipase, bilirubin and amylase levels, hyperuricemia, mild pain at the site of injection, increase in fibrinogen	< 2 µM	[208]
	MM	I/II	24	OR: 25 % (6/24) SD: 25 % (6/24)	Mild pain at the site of injection, hyperuricemia, elevated serum lipase, bilirubin, amylase and LDH, decreased hemoglobin, platelet and WBC count	< 2 µM	[52]
	HCC	II	76	OR: 3% (2/76) SD: 61% (50/76)	Transient and reversible encephalopathy, skin irritation, or discomfort at the site of injection combined with low-grade fever, decreased serum sodium, hemoglobin, albumin, fibrinogen levels, increased Potassium levels, uric acid and lipase	Undetectable	[51]
	MM	II	36	OR+SD: 28 % (10/36)	Discomfort at the injection site		[209]
	Melanoma	I/II	31	SD:31% (9/29) PMR: 27% (8/29)	Pain and rash at injection site, nausea, anorexia, pruritus, arthralgia	Undetectable	[210]

	MPM	II	39	PMR: 46% (18/39) SD: 31% (12/39)	Skin injection site reactions, neutropenia, anaphylactoid reactions, serum sickness	2 μM [$]	[64,211]
	HCC	III		Ongoing (NCT01287585)			
	Non-Hodgkin's Lymphoma	II		Ongoing (NCT01910025)			
	SLCL	II		Ongoing (NCT01266018)			
ADI-PEG20 plus Cisplatin	MM	I		Ongoing (NCT01665183)			
ADI-PEG20 plus Cisplatin and Pemetrexed	Arginine auxotrophic tumors such as MPM and NSCLC	I		Ongoing (NCT02029690)			
ADI-PEG20 plus Docetaxel	Solid Prostate and NSCLC tumors	I	18	PR: 6% (1/18) SD: 33% (6/18)		Undetectable	[212,213]
ADI-PEG20 Plus Doxorubicin	HER2 (-) Breast Cancer	I		Ongoing (NCT01948843)			
Peg-rhArgI	HCC	I	15	SD:26.7% (4/15)	Abdominal pain, diarrhea, nausea, elevated ALT, AST, GGT & bilirubin	< 8 μM	[141]
Peg-rhArgI plus Oxaliplatin and Capecitabine	HCC	II		Ongoing (NCT02089633)			

Peg-rhArgI (the second-line therapy after sorafenib)	HCC	II		Ongoing (NCT02089763)			

[#] Basal (Pre-treatment) level of arginine was ~ 130 µM
[$] Basal (Pre-treatment) level of arginine was ~ 63 µM
DCR- Disease-control rate (complete/partial response + stable disease)
SD- Stable disease
OS- Overall survival
PR- Partial response
CBR- Clinical benefit rate
MM- Metastatic melanoma
OR-Overall response (Complete + partial response)
CR- Complete response
PMR- partial metabolic response
MPM- Malignant Pleural Mesothelioma
Peg-rhArg1- Pegylated recombinant human arginase 1
ALT - Alanine Transaminase
AST - Asparate Transaminase
GGT - Gamma-glutamyl transferase
SLCL- Small Cell Lung Cancer
NSCLC- Non-Small Cell Lung Cancer
HER2- Human epidermal growth factor receptor 2

Table 3: Properties of arginine depriving enzymes

Arginine deiminase (E.C. 3.5.3.6)	**Arginase (E.C.3.5.3.1)**	**Arginine decarboxylase (E.C.4.1.1.19)**
Main products are citrulline and NH_3	Main products are ornithine and urea	Main products are agmatine and CO_2
At physiological pH, Mycoplasmal ADI is 300x more effective than arginase at depleting arginine	Very high alkaline pH optimum (pH 9.3) and has little enzymic activity at physiological pH	Mammalian ADC has a basic pH optimum (pH 8.23)
Circulatory half-life of ~ 4 h	Very short circulatory half-life (Approx. 30 minutes)	Not reported
Very high affinity for arginine (*K*m of 0.1-1 mM)	Low affinity for arginine (*K*m of 2-4 mM)	High affinity for arginine (*K*m of ~ 1mM)
Most normal cells and tissues are able to take up citrulline from the circulation	Ornithine can only be reconverted back into arginine in the liver and can cause toxicity to extra-hepatic tissues by inhibiting protein synthesis	Agmatine is not converted back to arginine under normal physiological conditions, may lead to its accumulation and toxicity to normal cells
Only found in microorganisms and is strongly antigenic in mammals	Human enzyme, non-immunogenic	Found in plants, microbes and human brain
Tumor sensitivity to ADI is dependent on ASS expression	The sensitivity of tumors to rhArg is independent of ASS expression	Studied only in human cervical cancer (HeLa) cell lines
Efficacious only in ASS-negative tumors	Efficacious in both ASS-negative and OTC-negative tumors	
No cofactor requirement	Mn^{2+} is essential for catalytic activity	Pyridoxal phosphate is a cofactor
Pegylation improves catalytic activity at physiological pH	Pegylation improves catalytic activity at physiological pH	PEGylation results in the total loss of catalytic activity